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ABSTRACT

Differential display was exploited to analyze expression patterns of premalignant (S2) and tumor (T4) cells, two sublines of a unique HMT3522 human breast cancer progression series, to identify candidate genes participating in the final-stage tumorigenic conversion in breast cancer. Message levels of a novel gene AZ-1 was at least 10-fold lower in T4 than in S2 cells cultured either on plastic or in a 3-D matrix system. AZ-1 was abundantly present in nonmalignant counterpart S1 cells and human breast luminal epithelial cells. In contrast, expression of this gene was drastically repressed in ten human breast epithelial cancer lines. Modulation of AZ-1 gene expression by phenotypic alteration of tumor cells was examined in a reversion system described recently in our laboratory. Briefly, when T4 cells were cultured in the presence of inhibitory β 1-integrin antibody in a 3-D system, they reverted morphologically to S1-like cells. They formed acini while re-assembled a basement membrane, re-organized cytoskeleton network, suppressed cyclin D1 and were growth arrested. Interestingly, AZ-1 gene was up-regulated in the reverted T4 cells to a level reminiscent of that seen in the S1 cells. Based on the sequence homology with functional coiled-coil domain of several structural proteins, i.e., myosin heavy chain and NuMA and based on the fact that AZ-1 is re-expressed in reorganized cells, AZ-1 may play a role in organization of cytoskeletal architecture.

INTRODUCTION

It is now widely accepted that extracellular matrix (ECM) is a key component of tissue microenvironment playing a determinant role in functional differentiation of developing and adult epithelia (1-3). In the mammary gland, extensive data exist showing that basement membrane components regulate the morphological and functional differentiation of mammary epithelial cells in culture and in vivo (4-6). In addition, altered interactions with ECM have been observed in mammary tumor development, emphasizing the importance of microenvironmental regulation in normal development and malignancy (7-9). Signals provided to mammary epithelium by basement membrane may be mediated by integrins, the transmembrane heterodimeric cell-surface receptors that link ECM to structural and functional elements within the cell (10-12). Several integrin receptors for laminin, the main component of basement membrane including α 1 β 1, α 2 β 1, α 3 β 1, α 6 β 1 and α 6 β 4 are expressed in normal human mammary epithelium. Altered expression of these receptors is a common occurrence in breast tumors (13-15). The disrupted tissue architecture observed in mammary adenocarcinoma is also frequently associated with alterations in integrin heterodimer profiles (16,17). Changes in β 1-, β 4-, α 2-, α 3- and α 6-intergrins have been reported for mammary tumor cell lines and in tissue sections, and were shown to be associated with tissue disorganization, loss of polarity, increased tumor aggressiveness, and

metastasis (18). Integrins are known to possess intrinsic kinase activity and to require associated molecules for signaling. The emerging concept is that integrin cooperatively works with linked kinase, or growth factor receptors, or also through their interactions with the cytoskeletal components. However, a relationship between altered signal transduction via integrins and the adherens junction pathways, and its relevance to the origin of the tumor phenotype has not been directly examined. This is mainly due to the lack of appropriate model systems in which such changes can be studied (19).

We have thus taken advantage of a unique epithelial cell model of breast cancer developed by Briand and coworkers (20,21). The HMT-3522 breast cancer series was established under chemically defined conditions from a breast biopsy of a woman with a nonmalignant breast lesion (22). The established cell line S-1 is entirely dependent on exogenous epidermal growth factor (EGF). In passage 118, cells were adapted to grow in medium without EGF and a new growth-transformed subline S-2 cells was generated and propagated at a high growth rate without exogenous EGF (23). A highly dramatic shift in phenotype was observed in passage 238 when the S-2 cells became tumorigenic in nude mice. After two mouse-culture passages, the resulting malignant transformed cell line (T4-2) was refractory to the growth-modulating effect of EGF and presented an extra copy of a chromosome marker, 7q- (21). These three cell lines (S-1, nonmalignant, S-2, premalignant, and T4-2, tumor) one originating from the other by spontaneous genetic events, therefore, provide a unique tool for addressing the carcinogenic event and particularly for us the altered ECM-signaling pathways through integrins involved in malignant conversion in the breast.

In addition to elucidating integrin signaling, we have also exploited molecular approach to search for candidate genes that might be involved in cellular transformation. Differential display originally described by Liang and Pardee (24) offers a powerful tool for this endeavor. The method is based upon comparison of mRNAs expressed in two or more cell populations by running their reverse transcribed and radioactively-labeled PCR products on sequencing gels in adjacent lanes. The bands revealing detectable differences between cell populations are cut and the cDNAs are eluted. After PCR re-amplification, the eluted cDNAs can be directly used as probes in northern blots for verification and in subsequent recovery of the full-length clones from cDNA libraries. By using suitably chosen PCR primers, the majority of the cDNAs can be displayed as 100-600 bp fragments. Several candidate tumor suppressor genes in breast cancer including $\alpha 6$ integrin were identified by this method (25).

To encompass a broader spectrum of the functional roles of tumor suppressors, our research interest is also focused on a known tumor suppressor gene Rb whose mutant alleles are seen in all retinoblastomas, most sarcomas, small cell lung carcinomas, and many breast and bladder carcinomas (26). Rb is a nuclear phosphoprotein that undergoes extensive phosphorylation in late G1, several hours before the onset of S phase, and remains in this hyperphosphorylated state through S, G2, and most of M phases. These phosphate groups are stripped off upon emergence from M phase. Phosphorylation of Rb seems to control its activity. This was initially indicated by the behavior of the DNA tumor virus oncoproteins,

which seem to be associated only with the underphosphorylated form of Rb. It was suggested that the hypophosphorylated form of Rb, found in G1, is functionally active in blocking growth. Accordingly, the hyperphosphorylated form of Rb in mid/late G1 leads to its functional inactivation, in turn permitting transit of the cell into late G1 and S phase (27). The interest in Rb springs from their ability to regulate cell cycle processes negatively, being able, for example, to dramatically slow down neoplastic growth. So far, among these genes, only Rb is firmly established to act as a tumor suppressor, because its lack-of-function is clearly involved in tumor onset and progression. It has been found deleted or mutated in most retinoblastomas and sarcomas, but its inactivation is likely to play a crucial role in other types of human cancers such as breast cancer (28).

RESULTS/EXPERIMENTAL METHODS

To address the main objectives of the grant proposal, we have explored the roles of ECM receptors (integrins) in morphological alterations in breast malignancy with effective functional blocking antibodies. The observations have been published recently (29) and the significant findings are described here. In addition, to take advantage of the human breast cancer progression series initially developed in Denmark by Briand and coworkers (21) and re-established in our laboratory, we have exploited differential display protocol to identify candidate tumor suppressor genes. Two isolated genes, one tentatively named AZ-1 is completely novel and the other is a member of the forkhead gene family of transcription factor. We have made major progress in cloning and characterization of AZ-1 gene and a manuscript is now being prepared to summarize the recent discovery (30). Finally, progress was also made on examining the phosphorylation status of a known tumor suppressor gene Rb and its link to breast tumor progression.

I. Integrin blocking antibody treatment served as a paradigm in the search of therapeutic remedy for breast cancer

A. Function blocking β 1-integrin antibody cause dramatic phenotypic reversion of the T-4 cells and growth arrest

In our previous annual progress communication, we have reported that tumorigenic T4-2 cells had both a higher total level of β 1-integrin and an elevated ratio of cell surface β 1- to β 4-integrins than the nonmalignant S-1 cells. To examine whether the aberrant malignant behavior may be a reflection of the changes in these integrins, we treated the T4-2 cells in 3-D with varying concentrations of a previously characterized rat monoclonal β 1-integrin antibody (clone A1IB2) which has been shown to inhibit ligand binding (31). This antibody caused massive apoptosis in S-1 cells, as shown previously (17), while T4-2 cells were refractory. Remarkably, however, in addition to resistance to apoptosis, almost all the antibody-treated T4-2 tumor cells assumed a morphology which was indistinguishable from that observed in S-1 cultures and was discernible as early as 4 d after incubation. To determine whether the

antibody-treated T4-2 cells have truly reverted to a "nonmalignant" phenotype, we cryosectioned the colonies and examined their morphology by immunofluorescence confocal microscopy. As markers of normal acinar formation, we examined both cytoskeletal organization and superimposition and distribution of cadherins and catenins. Sections of S-1 acini revealed uniform and polarized nuclei (stained with propidium iodide; red), well-organized filamentous actin (FITC phalloidin; green) (Fig. 1a), and uniformly superimposed E-cadherin and β -catenin at the lateral cell-cell junctions (Fig. 1b). In contrast, untreated or IgG-treated tumor cells had polymorphic nuclei and a grossly disorganized actin cytoskeleton, visualized as random, hatched bundles (Fig. 1a'). Additionally, E-cadherin and β -catenin were not colocalized (Fig. 1b'). In contrast, β 1-treated T4-2 cells (referred to as T4- β) revealed striking rearrangements of cytoarchitecture as demonstrated by their well-organized acini (Fig. 1a''), and cytokeratin 18 intermediate filament (not shown) networks. Furthermore, organized adherens junctions became evident in T4- β 1 acini (Fig. 1b') and were accompanied by the re-establishment of E-cadherin-catenin complexes (not shown). These changes were shown to occur in greater than 95% of the tumor colonies treated with blocking antibody, as quantified by analyzing the numbers of disorganized vs organized spheroids in relation to the S-1 and the mock-treated T4-2 cells (Fig. 1c).

To investigate whether the described phenotypic reversion is associated with cell cycle regulation, markers of proliferation and cell cycle status was examined in T4- β 1 cells. These cells showed a decrease in [3 H]thymidine incorporation and the size of the acini which was now composed of only 6-8 cells, similar to that observed for S-1 cells (Fig. 1, d and e). Cryosections of T4- β 1 colonies incubated with antibodies against either collagenIV (Fig. 1g') or laminin (not shown) revealed deposition of a basally distributed, almost continuous basement membrane, with characteristics similar to that observed in the S-1 acini. In contrast, punctate and inversely polarized collagen IV (Fig. 1g) and laminin immunostaining (not shown) were observed in the mock-treated tumor colonies. Thus, these tumor cells had retained the ability to deposit a basement membrane and to form polarized structures if the correct structural cues could be received. T4- β 1 colonies also had dramatically decreased cyclinD-1 levels again comparable to that seen in S-1 cultures (Fig. 1f) and markedly reduced Ki-67 levels (not shown). In addition, T4- β 1 cells revealed a drastic increase in the negative regulator of cell cycle p21 (not shown). From these results, it is suggested that most reverted cells had exited the cell cycle and therefore had a reduced propensity to proliferate.

B. Reduced tumorigenicity of β 1-inhibitory antibody treated T4-2 cells in nude mice

To find out whether phenotypic reversion of tumor cells would be sufficient to reduce tumorigenicity in vivo, we injected tumor cells treated in suspension with β 1-integrin blocking mAb, mock mAb, or no treatment for 3 h, as well as S-1 cells into nude mice. Within two weeks small nodules were observed in all injected sites including the S-1 controls (not shown). Whereas these nodules regressed rapidly in the S-1 and T4- β 1 groups, actively growing tumors were observed in greater than 75-90% of the mock mAb or vehicle-treated T4-2 mice. Upon sacrifice we observed both a significantly reduced tumor number and tumor size in the T4- β 1

group (Table I). These data suggested that "normalization" of the tumor cell phenotype in culture has a counterpart in vivo where the malignant potential is reduced or lost.

II. Identification of candidate tumor suppressor genes by differential display protocol

A. Isolation and characterization of a putative tumor suppressor gene AZ-1

In a 3-dimensional (3-D) culture system, we have previously shown that a cell's ability to properly sense signals from cell-extracellular (ECM) interactions was perturbed when breast cells became malignant (5). The unique epithelial cell model developed from HMT3522 breast cancer series was used to investigate the mechanism underlying the perturbations of ECM-mediated signal transduction events in breast tumorigenicity. The nonmalignant (S1), premalignant (S2) and tumor (T4-2) cells grown in a 3-D environment recapitulate the phenotypic and genotypic alterations of human breast cancer progression. To elucidate the molecular alterations in the final-stage of tumorigenic conversion, differential display was set up to compare the gene expression patterns between premalignant (S2) and tumor (T4) cells. We employed RNAimage kit (Genhunter, Inc) with arbitrarily-designed hexamer and anchored oligo d(T) primers as the PCR primers for amplification of the d(T)-primed cDNAs. Different intensities of radiolabelled messages representing relative abundance of various genes were displayed on a denaturing sequencing gel. Bands revealing greater than 10-fold differences in S2 and T4 samples were cut out, and the cDNAs were eluted and re-amplified. The cDNAs from the bands of interest were then used as probes in northern hybridization. Two of the isolated 200-300 bp cDNA fragments were shown by northern blot to annealed to messages that were expressed significantly higher in S2 than in T4 cells. The magnitude of differences in message levels are similar for RNAs isolated from S2 and T4 cells cultured on tissue culture plastic or embedded in ECM (Fig. 2A). The differential expression patterns of these two cDNAs match the classic definition of tumor suppressor genes and we have thus focused our research efforts on further characterizing their identities and functions.

The gene #1 (tentatively named AZ-1) has a message size of 4.4kb. The high abundance of AZ-1 gene was also observed in several nonmalignant breast cells including S1 (the non-malignant counterpart of S2), MCF10A, and luminal epithelial cells from normal human breast tissues. On the other hand, AZ-1 gene was either not detectable or expressed at a very low level in ten breast epithelial cells lines examined (Fig. 2B). Down regulation of AZ- in all breast cancer cells examined strongly suggested this gene might play a role in malignant transformation in breast tissues. In addition to the mammary tissues, a 4.4kb AZ-1 message was present in heart, brain, lung, kidney and pancreas. The same message size, however, was not found in liver, skeletal muscle and placenta. In collaboration with Drs, Colin Collins and Joe Gray's group (LNBL), fluorescence in situ hybridization (FISH) was used to localize AZ-1 gene to human chromosome 10, subband q26, a region which has been shown to be on tumor suppressive loci in prostate adenocarcinoma and glioblastomas (32,33).

Through the 5' RACE (rapid amplification of cDNA end) protocol, a 3.8-Kb cDNA fragment

of the AZ-1 gene has been assembled and sequenced. The AZ-1 cDNA fragment contains a 1713bp open reading frame encoding a predicted protein size of 64 kd. From the cDNA sequence information we have so far, it indicates that AZ-1 is novel at the nucleotide levels. Using a variety of homology search programs including BLAST (basic local alignment search tool), we found that AZ-1 shares high protein sequence homology in the coiled-coil domain with several protein families notably myosin heavy chain, NuMA (nuclear mitotic apparatus protein).

B. AZ-1 gene is re-expressed in phenotypically -reverted tumor cells

Since AZ-1 is homologous to myosin heavy chain and plakin family proteins that are known to be involved in organization of cytoskeletal architecture, we were interested in determining whether AZ-1 gene expression could be modulated by phenotypic alteration of tumor cells in a reversion system described in section I. Briefly, when T4 cells were cultured in the presence of inhibitory β 1-integrin antibody in a 3-D assay system, they reverted morphologically to S1-like cells. They formed acini while re-assembled a basement membrane, re-organized cytoskeletal network, suppressed cyclin D1 and were growth arrested. Interestingly, AZ-1 gene was up regulated in the reverted T4 cells (analogous to p21) to a level reminiscent of that seen in the S1 cells (Fig. 2C). The recurrence of AZ-1 in normalized breast tumor cells could be linked to its putative function in cytoskeletal reorganization. However, further studies will need to confirm this hypothesis.

C. A forkhead family protein as a candidate tumor suppressor gene

The gene #2 also displayed marked differential expression pattern and it has a message size of 6.7kb. From the sequence data we have in hand, the gene#2 was clearly shown to be a transcription factor since it contains homologous sequence to the consensus DNA binding domain of forkhead protein family. Progress has been made to further characterize the entire gene structure and to dissect the DNA binding and activation domains.

III. Analysis of the tumor suppressor gene Rb in HMT-3522 progression series

Similar to the published results using other cell models, we also observed both hypophosphorylated and hyperphosphorylated forms of Rb in exponentially growing S-1 cells whereas only hypophosphorylated Rb was seen in growth arrested cells. At day 10 in 3-D culture when S-1 cells have undergone morphogenesis, Rb is only present in hypophosphorylated form. In contrast, after the same period of time in 3-D, the pre-malignant S-2 and tumor T4-2 cells showed both hypo- and hyperphosphorylated forms of Rb indicating that modulation of functional Rb is linked to tumorigenicity (Fig. 3A). To further demonstrate the phosphorylation status of Rb is a marker for breast malignancy, we examined the alterations in status in tumor cells treated with blocking β 1-integrin antibody. Interestingly as we have predicted, the reverted T4B1 cells which have been shown to undergo growth arrest and morphogenesis revealed only the hypophosphorylated form of Rb (Fig. 3B). These results

clearly suggested that proper regulation of Rb is crucial for breast epithelial cells to maintain normalcy.

CONCLUSIONS

In this report, we have described the exciting results of using inhibitory $\beta 1$ -integrin antibody to phenotypically revert breast tumor cells to normal-like cells. This reversion system complements to our HMT 3522 progression series that reciprocal nonmalignant to tumor conversion could be studied. In addition, we have discovered a novel putative tumor suppressor gene AZ-1 whose expression is clearly modulated by the malignancy status of breast epithelial cells. We are currently investigating whether modulation of AZ-1 gene expression is cell-cycle dependent like most of known tumor suppressor genes. In addition, we are making different expression constructs for use in antibody production, cellular localization and stable transfection. Our future plan is able to use AZ-1 as a marker gene to detect breast tumor progression. Further studies will also focus on nuclear localization of Rb and its link to nuclear matrix components such as NuMA (nucleus mitotic apparatus protein) and laminB. All the information we gather here provides a molecular and cellular system for our future understanding of mechanisms of breast tumor development.

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APPENDIX (publications and abstracts from research progress from Oct. 1, 1996 - Oct. 15, 1997)

1. Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C and Bissell MJ. 1997. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. J. Cell Biol. 137:231-245.
2. Chen HM, Petersen OW, S. Mian and Bissell MJ. 1997. A novel putative tumor suppressor AZ- 1 and its plausible role in cytoskeletal reorganization. Manuscript in preparation.
3. Chen HM, Weaver VM, Wang F, Petersen OW, and Bissell MJ. 1997. A novel gene lost when human breast cells become malignant could serve as a progression marker. Abstract submitted for DOD Era of Hope meeting, Oct.31- Nov.4, Washington DC.
4. ChenHM, Petersen OW, and Bissell MJ. 1997. A novel gene lost when human breast cells become malignant is reexpressed in reverted cells. Abstract submitted for ASCB meeting, Dec.13-17, Washington DC.

FIGURE LEGEND

Figure 1. β 1-inhibitory antibody treatment of tumor cells leads to the formation of reverted acini. (a-a') Confocal fluorescence microscopy images of F actin: Both the S-1 (a) and T4- β 1 reverted acini (a') showed basally localized nuclei (propidium iodine) and organized filamentous F-actin (FITC), while T4-2 mock-treated colonies (T4-2 IgG) had disorganized, hatched bundles of actin and pleiomorphic nuclei (a'). (b'-b'') Confocal immunofluorescence microscopy images of E-cadherin (FITC) and β -catenin (Texas red): In S-1 (b) and T4- β 1 reverted acini (b''). E-cadherin and β -catenins were colocalized and superimposed at the cell-cell junctions. (c) Quantitative analysis of tumor cell conversion efficiency by β 1-integrin function blocking antibodies: Greater than 95% of S-1 and T4- β 1 colonies were scored as organized, while 97% of T4-2 IgG colonies were considered disorganized. Other reversion criteria, such as scoring for actin and cadherin organization also yielded comparable results (not shown). (d) Cell number per colony in S-1 acini, T4-2 IgG colonies and T4- β 1 acini from 3-5 experiments: Both S-1 and T4- β 1-revertant acini contained 6-8 cells, while T4-2 IgG tumor colonies contained 18-22 cells when scored after 10-12 d (d). (e) Percent of thymidine-labelled cells in S-1, T4-2, T4-2 IgG, and T4- β 1 colonies: While a high percentage of T4-2 IgG colonies (greater than 30%) were still actively growing, the T4-2 β 1 revertant acini had a greatly reduced growth rate similar to that observed in the S-1 acini. (f) Immunoblot of cyclin D1 levels in S-1, T4-2, T4-2 IgG, and T4- β 1 colonies: The level of D1 cyclin was clearly decreased to the level of S-1 acini after β 1 inhibitory antibody treatment. (g and g') Collagen IV deposition as an indicator of basement membrane organization: T4- β 1 reverted acini (g') deposited an organized collagen IV-containing BM at the cell-ECM junctions, similar to that observed in S-1 acini. Note the contrast with T4-2 mock-treated tumor colonies (g). All cultures were analyzed after 10-12 d inside EHS. Bars: (a-a' and b'-b'') 16 μ m; (g and g') 25 μ m.

Figure 1/Chen

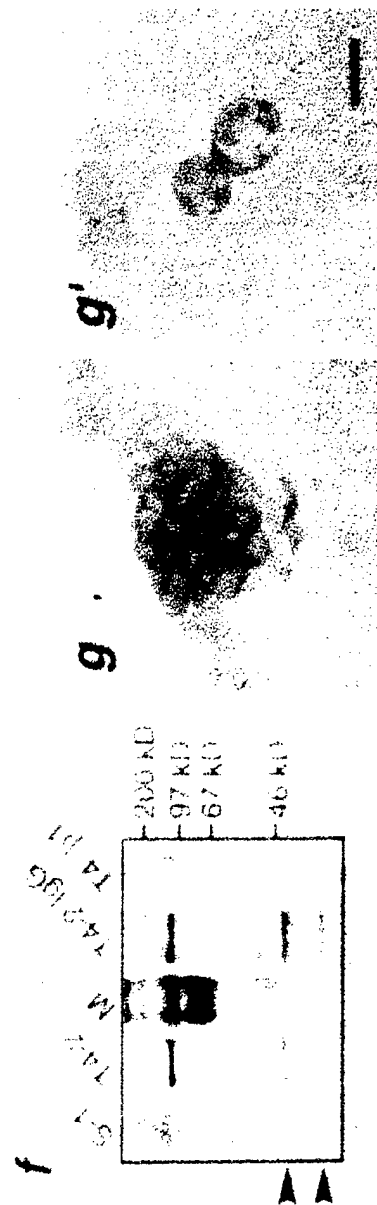
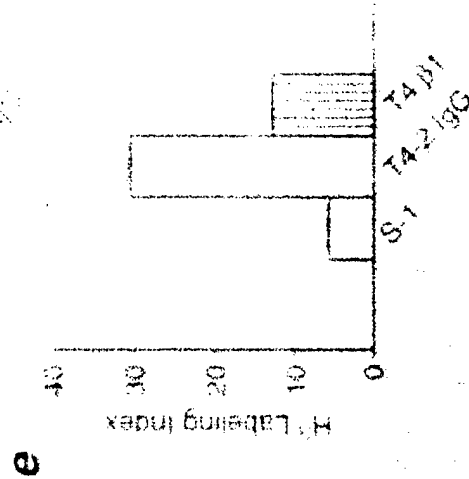
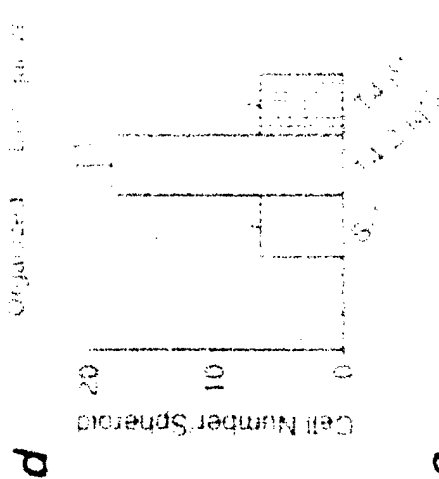
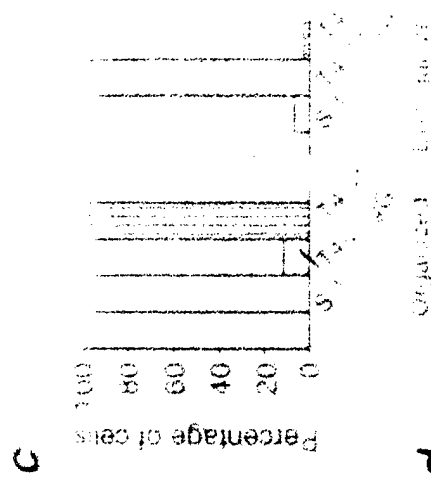
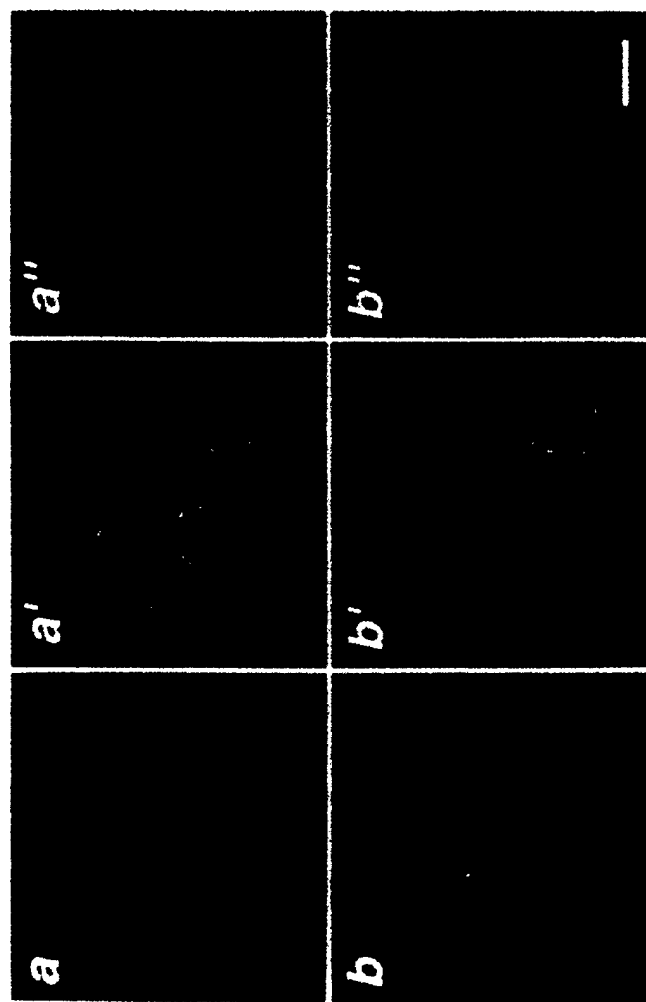


Figure 2/Chen AZ-1, a new putative class II tumor suppressor gene isolated by differential display

A

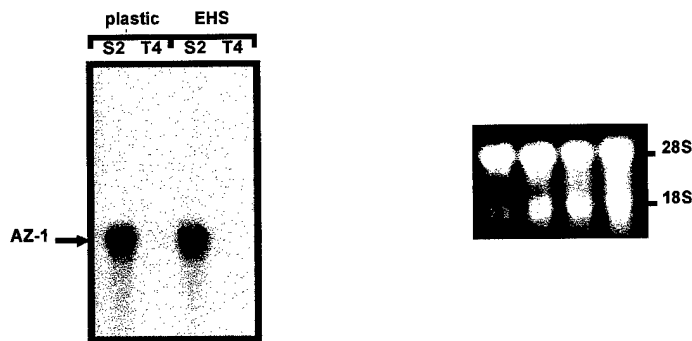


FIGURE 2A. Differential expression pattern of AZ-1 gene in premalignant (S2) and tumor (T4-2) cells. Twenty μ g of total RNAs from S2 and T4-2 cells grown on tissue culture plastic and in EHS matrix gel were loaded on a formaldehyde agarose gel. The RNAs were transferred and the blot was hybridized with 32 P-labelled 3.2 kb AZ-1 cDNA probe. Left panel: the radiogram after 24 h exposure. Right panel: the ethidium bromide stain of formaldehyde gel. A 4.4 kb AZ-1 message is shown in S2 cells grown on tissue culture plastic and in EHS matrix gel. Under identical conditions, AZ-1 message is absent in tumor cells.

B

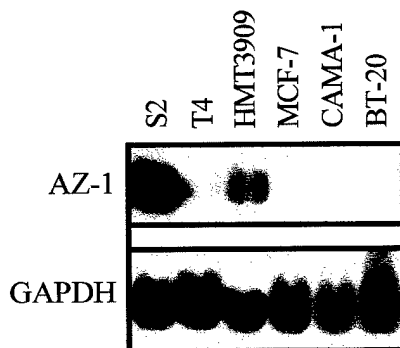


FIGURE 2B. Down regulation of AZ-1 gene in malignant breast epithelial cell lines. Twenty μ g of total RNAs isolated from S2 and ten malignant breast epithelial cell lines, i.e., T4-2, HMT3909, MCF-7, CAMA-1, BT-20 (data shown), MDA468, SKBR3, T47-D, MDA231, HS578T, and BT549 (data not shown) were analyzed as described in Fig.C.1. Except for HMT3909, AZ-1 message was undetectable in all other malignant breast epithelial cells after 24 h exposure. It is interesting to note that HMT3909 is not highly malignant (indeed it often does not give rise to tumors; in our assay it behaves like S2 cells); it also appears to have myoepithelial character. A GAPDH cDNA probe was used to control loading.

C

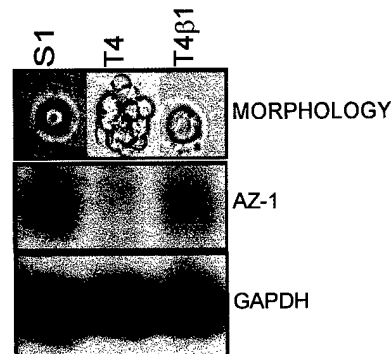


FIGURE 2C. Re-expression of AZ-1 gene in T4-2 revertants treated with blocking β 1-integrin antibody. RNAs were isolated from S1 and T4-2 treated with β 1-inhibitory integrin (T4 β 1) or mock-antibody (T4); for experimental details of reversion see Weaver et al., 1997. Twenty μ g of total RNAs were analyzed. A GAPDH cDNA probe was used to reveal an equal loading. Top panel: morphology of S1, T4 and T4 β 1 cells in 3D matrix.

Figure3/Chen

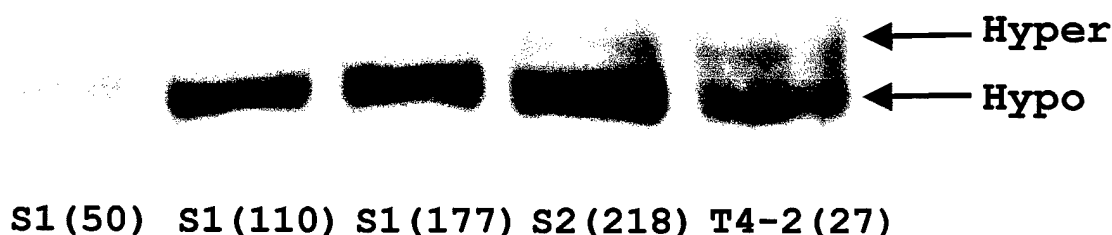


Figure 3A. Modulation of functional Rb is linked to tumor progression in HMT 3522 series. Western blot of Rb protein was analyzed with total cell extract (Laemmli) prepared from S1(50), S1(110), S1(117), S2(218) and T4-2 cells of the HMT-3522 progression series. Cells were cultured for 10 days in 3D matrigel culture. Hyperphosphorylated (Hyper) and hypophosphorylated (Hypo) forms of Rb are indicated. The doublet of hypophosphorylated and hyperphosphorylated Rb appears in the pre-malignant stage of cancer progression.

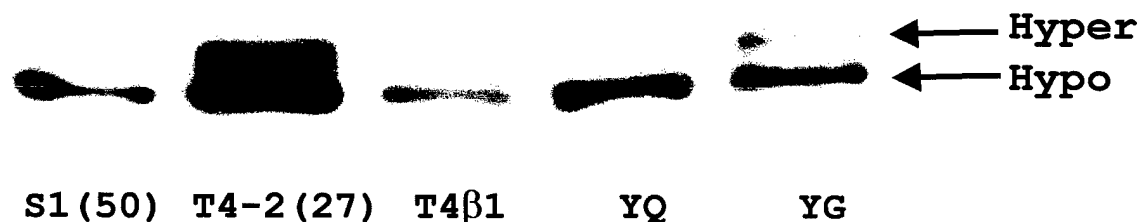


Figure 3B. Dephosphorylation of Rb in T4β1-reverted cells. Western blot of Rb protein was analyzed with total cell extract (Laemmli) prepared from S1(50), T4, and T4-β1 reverted cells (details see Results and Experimental Methods section I). Growth arrested (YQ) and exponentially growing (YG) cells were used as controls. Hyperphosphorylated (Hyper) and hypophosphorylated (Hypo) forms of Rb are indicated. Similar to the S1(50) cell, only the hypophosphorylated form of Rb is expressed in the T4-β1 reverted cells.

Table 1. Effect of β 1-Inhibitory Antibody Induced Phenotype Reversion on Tumorigenicity In Vivo

Treatment description	Total number of tumors per group	Tumor size		
		Large tumors*	Small tumors [†]	Mice with no tumors
Control nontreated T4-2 cells	15/16	14/16	1/16	1/16
Non-immune rat IgG-treated T4-2 cells	14/16	11/16	3/16	2/16
β 1-integrin function blocking antibody-treated T4-2 cells	7/16	5/16 [§]	2/16	9/16 [§]

*tumors $>5 <300 \text{ mm}^3$.

[†]tumors $<5 \text{ mm}^3$.

[§] $P < 0.005$ by χ^2 test.